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# Precocious induction of vitellogenin with JH III in the twospotted stink bug, *Perillus bioculatus* (Heteroptera: Pentatomidae)☆

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**Abstract**

The effect of juvenile hormone (JH) III on the hemolymph composition of vitellogenin was examined in *Perillus bioculatus*. Adult females were treated topically with JH III, and the premature presence of vitellogenin in the hemolymph was then detected using electrophoresis and Western blot analyses. JH III treatment resulted in a dose-dependent early production of vitellogenin that was detectable 48 h before vitellogenin was present in non-treated insects. Vitellogenin was not observed in the hemolymph of JH III-treated adult males. The techniques reported here may be useful for the detection, isolation and characterization of compounds with JH-like activity in *P. bioculatus* and other species of Heteroptera (which are thought to have JH-like substances other than the JHs with known chemical identity). These same techniques may also provide a method for researchers to investigate the interactions of JH-like compounds and other substances, such as ecdysteroid, in the regulation of vitellogenesis in Heteroptera.
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*Keywords:* Bioassay; Reproduction; Predator; Induction; Heteroptera; Juvenile hormone; Vitellogenesis; Vg

## 1. Introduction

In insects, the process of vitellogenesis, biosynthesis, distribution and packaging of the yolk protein precursor known as vitellogenin (Vg), is under hormonal control, and includes ecdysteroids and juvenile hormones (JHs) (Gäde et al., 1997). In Heteroptera, vitellogenesis is primarily regulated by JH, with the role of ecdysone considered to be less significant (Davey, 1997). JHs are a group of acyclic sesquiterpenoids secreted by the corpora allata that regulate many aspects of reproduction, as well as other aspects of insect physiology, including metabolism, development, metamorphosis and diapause (Simonet et al., 2004). JH has been shown to stimulate Vg biosynthesis in female fat body and follicle cells and uptake from the hemolymph by oocytes in the growing follicles (Nation, 2002). While the chemical identity of several JHs has been determined, isolation and characterization of JH in Heteroptera has not been accomplished, although evidence suggests that it is different from the JH of other insect orders (Kotaki, 1996; Davey, 1997).

Recently, vitellogenesis has been developed as a biomarker to monitor effects caused by endocrine-disrupting agents (Hahn et al., 2002). In many insects, there is a direct relationship between the synthesis of Vg and the presence of JH. Therefore, with the discovery of the anti-allatal compounds precocenes, bioassays were developed in which a chemical allatectomy was followed by treatment with compounds that were assessed for their ability to restore JH activity (Venugopal and Kumar, 2000). Unfortunately, one heteropteran species, *Perillus bioculatus* has proven to be detrimentally sensitive to treatment with low levels of precocenes, causing ca. 50% death and neurological abnormalities

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in the surviving adults (unreported data), leading us to search for other means in which to study JH-like activity in this insect.

*P. bioculatus* is a native North American predator of the Colorado potato beetle and is a proven beneficial predator used in augmentative biological control programs (Hough-Goldstein et al., 1996). The hemolymph of female *P. bioculatus* contains a major vitellogenin with a molecular mass of 528 kDa that consists of 3 apoproteins with masses of 177, 84 and 59 kDa. (Adams et al., 2002). Vg levels in the hemolymph start to increase in females 2.5 days after adult emergence and reach peak levels at 5.5 days after adult emergence (Adams et al., 2002). Treatment of female *P. bioculatus* adults with JH III has been shown to increase the amount of vitellogenic protein detected in the ovaries, and supported the conclusion that the JH III treatment positively affected vitellogenin synthesis (Adams, 1997; Adams et al., 2002). In addition, extended treatment of larval stages of the dipteran, *Chironomus thummi* resulted in a small number of individuals initiating vitellogenesis precociously in the subsequent pupal stage (Laufer et al., 1986). Therefore, it seemed probable that treatment of *P. bioculatus* with JH III might cause a change in the timing as well as the amount of production of vitellogenin. The purpose of this study was to determine if such a change occurred and if that change would be suitable to assist in the investigation of hormonal regulation of vitellogenesis, tissue competency and characterization of JH-like compounds in *P. bioculatus.*

## 2. Materials and methods

### 2.1. Insect rearing

The *P. bioculatus* originated from a colony at the USDA-APHIS PPQ-MPPC (Mission, TX, USA) and were maintained at the Biological Control of Insects Research Laboratory (Columbia, MO, USA) for ca. 30 generations. Experimental rearing conditions were 25±2 °C, 45±10% RH, and a photoperiod of 16:8 (L:D) h, maintained in a walk-in growth chamber. Groups of ca. 400 eggs, defined as a cohort, were obtained over 4 days from approximately 240 females of a colony maintained on 2nd and 3rd instar larvae of *Trichoplusia ni*. First and second instar nymphs were reared in groups of ca. 300 within clear 2.4 l round plastic containers fitted with a filter paper liner, rolled 20 cm × 18 cm vinyl netting, moist dental wicking and covered with cheesecloth. Fresh 2nd and 3rd instar larvae of *T. ni* were added daily. Third through fifth instar nymphs were reared in similar containers, but at a density of 20 per container and fed 3rd instar larvae of *T. ni.* Preliminary data from test insects showed that eggs hatched in 5–6 days, nymphs molted every 2–4 days for second and fifth instar nymphs, respectively. Additionally, the survival to adult was ca. 87%. Late fifth instar nymphs were observed every 2–4 h and adult eclosion (separating from the nymphal exuviae) was recorded. Individuals were sexed at adult emergence, and females were randomly selected from each cohort to be used for control or JH treatments. After treatment, adult females were isolated in half pint paper containers lined with filter paper, containing moist dental wicking, 3rd instar *T. ni*, and covered with a Petri dish lid.

### 2.2. Bioassay

Late fifth instar and newly emerged female adults that did not have a hardened cuticle (within 4 h of adult eclosion) were used for the treatments. Insects were anesthetized with $CO_2$ for no longer than 1 min. JH treatments and acetone controls were applied topically on the cuticle surface in various locations. JH III or JH I (Sigma-Aldrich Chemical Co., St. Louis, MO, USA and SciTech, Czech Republic, respectively) was dissolved in acetone at a dosage of 0.5 to 20 μg (1 to 3 μl total volume) per insect. Ten insects were treated with the same dose, and each experiment was repeated at least twice with a separate cohort of insects. Observations of movement, feeding and mortality were made every 4 h following treatment for all individuals in order to assess insect viability. Hemolymph was collected at 12 h intervals ranging from 12 to 48 h after adult emergence.

### 2.3. Sample collection

The meso- and metathoracic legs were severed at the coxal base of an anesthetized adult, followed by light pressure applied to the abdomen, before hemolymph samples of 1–10 μl were collected in a microcapillary pipette held at the point of amputation. The hemolymph was transferred to chilled microtubes, the volume recorded, fast frozen in liquid nitrogen, and stored at −80 °C. Samples were centrifuged at 10,000 × *g* for 10 min at 4 °C to remove hemocytes and other tissue fragments before analysis.

### 2.4. Electrophoretic procedures

Individual hemolymph samples were analyzed under denaturing conditions with seven percent acrylamide sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using the mini-Protean® 3 electrophoresis system (Bio-Rad, Hercules, CA, USA). One microliter of hemolymph was placed into 9 μl of purified water and mixed with 10 μl of loading buffer (0.042 M Tris pH 6.8, 4.2% glycerol, 4.2% 2-mercaptoethanol, 2% SDS, 0.002% Bromophenol Blue). The mixture was incubated at 100 °C for 10 min before loading onto the gel. Samples were run at 120 V for 1.5 h. For staining of the separated proteins, the gel was incubated for 10 min in staining solution (0.05% Coomassie® Brilliant Blue R250 (Biorad), 50% methanol, 10% acetic acid) followed by overnight incubation in destaining solution (7% methanol, 5% acetic acid).

For Western blot analysis, duplicate gels were run with 0.2 μl of each hemolymph sample, and the proteins were

transferred from the gel to a nitrocellulose membrane (Optitran BA-S83, Schleicher and Schuell, Keene, NH, USA) by a semi-dry technique using the Trans-Blot® SD semi-dry transfer cell (Bio-Rad). The membranes were blocked with 1% bovine serum albumin (Sigma Chemical Co.) in Tris-buffered saline, probed with rabbit anti-vitellogenin polyclonal antisera (kindly provided by T.S. Adams) diluted 1 : 1000 in PBST (phosphate buffered saline with 1% Tween 20 (BioRad)), followed by an incubation with secondary antibody, alkaline phosphatase-conjugated anti-rabbit immunoglobulin (Sigma-Aldrich) diluted 1 : 1000 in PBST. The membranes were visualized colorimetrically using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT, Pierce Biotechnology, Inc., Rockford, IL, USA) as a substrate. Western blots and stained gels were imaged and densitometric analysis was performed using the Fluor-S™ MultiImager system with Quantity One® software (Bio-Rad). The densitometric fold increase was calculated by dividing the densitometric value of the 177 kDa band (in Optical Density Units × mm) by the value obtained in the same region of the gel in the matching control lane.

## 3. Results

The results of preliminary studies confirmed the following: JH III application to adult males did not result in Vg production (Fig. 1); no response was observed in the time of occurrence of vitellogenin in the hemolymph of adult females that had been treated with JH III as fifth instar nymphs (data not shown); treatment of adult females with JH III did cause a premature increase in the occurrence of vitellogenin in their hemolymph; and the dorsal abdominal segments just beneath the wing pads on adult females were found to be the most sensitive location for topically administering JH III, yielding the most consistent results. All data reported here resulted from the treatment of newly emerged adult females in that area.

Initial experiments focused on the response of adult females treated with 20 μg of JH III at adult emergence in order to determine the timing of the premature occurrence of vitellogenin. Hemolymph was collected at 12 h intervals ranging from 12 to 48 h following treatment. Analysis of the hemolymph proteins is shown in Fig. 1. Each lane depicted is of a hemolymph sample from an individual that is representative of each treatment group. No change in the occurrence of Vg resulted from acetone treatment. A gradual increase in Vg was detected over time in the females treated with 20 μl JH III, beginning with the 12 h time point (Fig. 1A). All 3 Vg apoproteins were detected (arrows), however the 177 kDa apoprotein was present in the greatest amount, which is consistent with previous findings (Adams et al., 2002). Hemolymph from a 10 d-old adult female was used as a positive control (CF). Western blot analysis using antisera to *P. bioculatus* Vg confirmed the presence of Vg (Fig. 1B) in female samples and also confirmed the absence of Vg in male samples. Treatment with JH I also resulted in early production of Vg, although in reduced amounts compared to the JH III treatments (data not shown).

The greatest response in premature production of Vg was detected at 48 h after adult emergence. Therefore, a set of experiments was conducted to determine what changes in the response of Vg production at 48 h occurred as a result of various doses of JH III applied to females at adult emer-

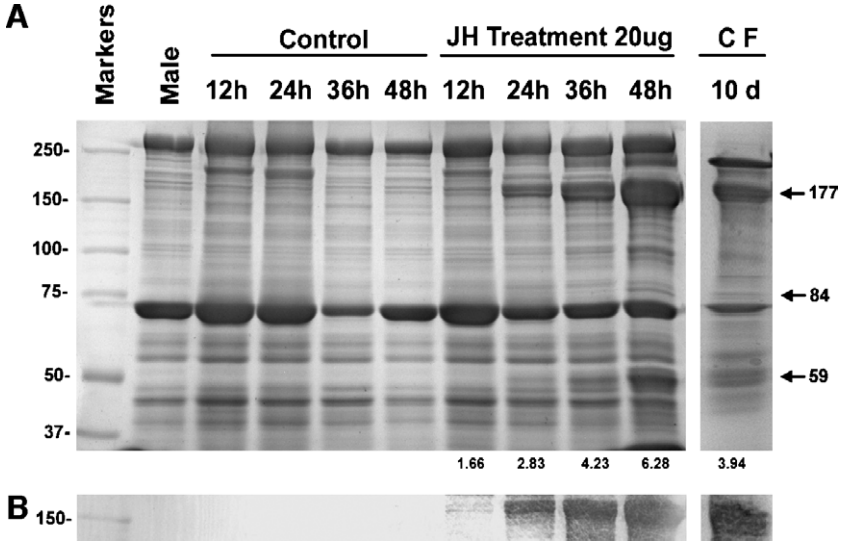


Fig. 1. Coomassie-stained SDS-PAGE (A) and Western blot (B) analyses of individual hemolymph samples collected at 12 h time intervals after adult emergence from females treated at adult emergence with control acetone or 20 μg JH III. Male hemolymph is shown as a negative control, and 10 d-old adult female hemolymph is shown as a positive control (CF). Arrows indicate the 3 vitellogenin apoproteins. The numbers below the lanes indicate the densitometric fold increase over control of the 177 kDa protein band.

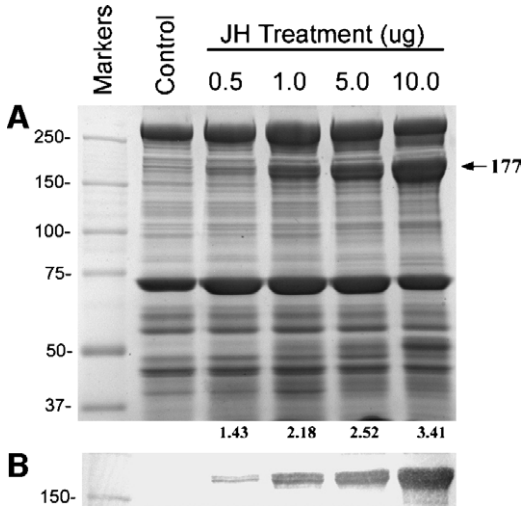


Fig. 2. Coomassie-stained SDS-PAGE (A) and Western blot (B) analyses of individual hemolymph samples collected 48 h after adult emergence from females treated at adult emergence with control acetone or 0.5–10 μg JH III. The numbers below the lanes indicate the densitometric fold increase over control of the 177 kDa protein band (arrow).

gence. The results demonstrated that the production of Vg in the hemolymph was JH III dose-dependent, as shown in Fig. 2. Each lane depicts hemolymph from an individual that is representative of each treatment group. A Vg response was detectable following as little as 0.5 μg JH III and increased in response to increasing doses up to 10 μl of JH III. Relative quantity of the 177 kDa band in comparison to all hemolymph proteins increased from 4.4% in the 0.5 μg treatment to 16.6% in the 10 μg treatment. Again, Western blot analysis confirmed the presence of Vg (Fig. 2B). Interestingly, the amount of protein present in the >250 kDa band (putative lipophorin based on molecular mass and abundance) changed very little with increasing JH treatment (Fig. 2A).

## 4. Discussion

A noticeable gap in our understanding of endocrinology in insects is the lack of characterization of substances with JH-like activity in Heteroptera (Davey, 1997). Thus far, most information results from the treatment of heteropteran species with JHs characterized from other insect orders. In contrast, considerable information is available on the structures of JHs in Lepidoptera, Coleoptera and Diptera. The approach described here has been developed, in part, because of the limitations associated with existing bioassays used to characterize substances with JH-like activity, and has been tested on *P. bioculatus* to demonstrate the application on a species of Heteroptera.

Our results show that a single treatment of newly emerged *P. bioculatus* adults with JH I or JH III caused a premature production of Vg in the hemolymph of the treated females, which was detectable up to 48 h prior to the time females normally begin production of Vg, but did not cause the appearance of Vg in the hemolymph of treated males. The premature production of Vg in the hemolymph of adult females was dose-responsive and detectable at levels as low as 0.5 μg of JH III. It should be noted that the observed response to JH III is likely to be much less sensitive than what would be expected for the endogenous JH of this insect.

Vg gene expression is known to be controlled in a sex-, stage- and tissue-specific manner (Cymborowski, 1992; Davey, 1997). The premature production of Vg that resulted in treated adult females demonstrated competency of the fat body at adult emergence and indicates that in non-treated insects either the titer of endogenous JH-like substance was too low to stimulate Vg production at that time, or possibly that another endogenous substance, such as ecdysone, inhibited either the production of the JH-like substance or inhibited Vg synthesis by the fat body. Since JH treatment of 5th instars did not result in Vg production, either the fat body had not yet developed competency to produce Vg or an endogenous substance at this stage interfered with the actions of JH.

Several bioassay systems have been developed to detect the presence of JHs or compounds with JH-activity. In most of these bioassays, the test chemical or extract is applied topically or injected into a developing insect larvae or pupae, and the anti-metamorphic effects caused by the treatment are assessed on the pupa or adult cuticle following development (Staal, 1972; Cymborowski, 1992). The *Galleria* wax test and the *Manduca* black mutant assay are both used to confirm JH activity (De Loof and Van Loon, 1979; Rankin and Riddiford, 1978) and can be sensitive for the quantitation of JH to below 2.6 pg (Engelmann and Mala, 2000). However, these bioassays are laborious and often require the maintenance of an additional insect colony and several days to complete. While not as sensitive as the classical bioassay methods, the approach outlined here may be more practical for screening multiple compounds for the identification and purification of substances with JH-like activity, as well as in the search for new compounds with direct anti-JH activity. Additionally, survival was permissible following surface treatment with JH I and JH III of both immature and adult stages, suggesting this method may prove valuable for further competency studies, and also indicated that the premature production of Vg was not a lethal effect.

Several species of Heteroptera that have been examined show stimulation of vitellogenesis by JH (Wyatt and Davey, 1996), although the structure of the substance(s) with JH-like activity within Heteroptera is unknown. Therefore, it is possible that this system would have application in other insect species as well. This is especially true because detection methods (e.g. Western blot technology) for measuring Vg in several insects are now available. Also, because this approach causes premature production of Vg, at a time when Vg would not normally occur (in contrast to increasing the

amount of Vg at a time when Vg already occurs), this method should work regardless of the oogenetic cyclicity of the insect.

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## References

Adams, T.S., 1997. Arthropoda–Insecta. In: Adams, T.S. (Ed.), Reproductive Biology of Invertebrates. Progress in Reproductive Endocrinology. J. Wiley & Sons, New York, pp. 277–335.

Adams, T.S., Filipi, P.A., Yi, S., 2002. Effect of age, diet, diapause and juvenile hormone on oogenesis and the amount of vitellogenin and vitellin in the twospotted stink bug, *Perillus bioculatus* (Heteroptera: Pentatomidae). J. Insect Physiol. 48, 477–486.

Cymborowski, B., 1992. Insect Endocrinology. Elsevier Press, New York.

Davey, K.G., 1997. Hormonal controls on reproduction in female Heteroptera. Arch. Insect Biochem. Physiol. 35, 443–453.

De Loof, A., Van Loon, J., 1979. A redescription of the *Galleria* bioassay for juvenile hormone and compounds with juvenile hormone activity. Ann. Soc. R. Zool. Belg. 109, 19–28.

Engelmann, F., Mala, J., 2000. The interactions between juvenile hormone (JH), lipophorin, vitellogenin, and JH esterases in two cockroach species. Insect Biochem. Mol. Biol. 30, 793–803.

Gäde, G., Hoffmann, K.H., Spring, J.H., 1997. Hormonal regulation in insects: facts, gaps, and future directions. Physiol. Rev. 77, 963–1032.

Hahn, T., Schenk, K., Schulz, R., 2002. Environmental chemicals with known endocrine potential affect yolk protein content in the aquatic insect *Chironomus riparius*. Environ. Pollut. 120, 525–528.

Hough-Goldstein, J., Janis, J.A., Ellers, C.D., 1996. Release methods for *Perillus bioculatus* (F.), a predator of the Colorado potato beetle. Biol. Control 6, 114–122.

Kotaki, T., 1996. Evidence for a new juvenile hormone in a stink bug, *Plautia stali*. J. Insect Physiol. 42, 179–186.

Laufer, H., Vafopoulou-Mandalos, X., Deak, P., 1986. Ecdysteroid titres in *Chironomus* and their relation to hemoglobins and vitellogenins. Insect Biochem. 16, 281–285.

Nation, J.L., 2002. Insect Physiology and Biochemistry. CRC Press, New York, pp. 425–451.

Rankin, M.A., Riddiford, L.M., 1978. Significance of haemolymph juvenile hormone titer changes in timing of migration and reproduction in adult *Oncopeltus fasciatus*. J. Insect Physiol. 24, 31–38.

Simonet, G., Poels, J., Claeys, I., Van Loy, T., Franssens, V., De Loof, A., Vanden Broeck, J., 2004. Neuroendocrinological and molecular aspects of insect reproduction. J. Neuroendocrinol. 16, 649–659.

Staal, G.B., 1972. Biological activity and bioassay of juvenile hormone analogs. In: Menn, J.J., Beroza, M. (Eds.), Insect Juvenile Hormones, Chemistry and Action. Academic Press, pp. 69–94.

Venugopal, K.J., Kumar, D., 2000. Role of juvenile hormone in the synthesis and sequestration of vitellogenins in the red cotton stainer, *Dysdercus koenigii* (Heteroptera: Pyrrhocoridae). Comp. Biochem. Physiol. B 127, 153–163.

Wyatt, G.R., Davey, K.G., 1996. Cellular and molecular actions of juvenile hormone: II. Roles of juvenile hormone in adult insects. In: Evans, P.D. (Ed.), Advances in Insect Physiology, vol. 26. Academic Press, pp. 1–155.